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Binding of HMG-I(Y) Elicits Structural Changes in a Silencer of the Human β-Globin Gene

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Proteins involved in repression of the human β-globin gene may be useful in the treatment of sickle cell anemia, in conjunction with therapy to reactivate fetal globin genes. If there is a reciprocal elevation of γ-globin expression upon repression, this approach could be useful in additional hemoglobinopathies. We previously showed that repression of the β-globin gene appears to be mediated through two DNA sequences, silencers I and II, and identified a protein termed BP1 which binds to both silencer sequences. In this study, we cloned two cDNAs encoding proteins which bind to an oligonucleotide in silencer I containing a BP1 binding site. These cDNAs correspond to HMG-I and HMG-Y, isoforms regarded as architectural proteins. We demonstrate that binding of HMG-I(Y) to this ogligonucleotide causes bending/flexure of the DNA. HMG-I(Y) also binds to a second oligonucleotide containing a BP1 binding site located in a negative control region upstream of the δ-globin gene, suggesting a role for HMG-I(Y) in repression of adult globin genes. Expression studies revealed that HMG-I(Y) is ubiquitously expressed in human tissues that do not express β-globin, being present in 48 of 50 tissues and six hematopoietic cell lines examined. Furthermore, HMG-I(Y) expression is down-regulated during differentiation of primary erythroid cells. We present a model in which HMG-I(Y) alters DNA conformation to allow binding of repressor proteins, and in which the relative amount of HMG-I(Y) helps to determine the repressive state of the β-globin gene. Am. J. Hematol. 60:27–35, 1999. © 1999 Wiley-Liss, Inc.

Key words: beta globin; repressors; HMG-I(Y)

INTRODUCTION

Although mutations in the human β-globin gene can cause a number of genetic diseases, such as sickle cell anemia (SCA), the mechanism of developmental regulation of this gene is not well understood. The β-globin gene is one of five globin genes clustered on chromosome 11. These genes, differentially expressed during development, include the embryonic (ε), fetal (Gγ and Aγ), and adult (δ and β) genes. During the time of embryonic or fetal gene expression, the adult β- and δ-globin genes are repressed. In contrast to the identification and understanding of the role of transcriptional activators that bind to DNA sequences near the β-globin gene, little is known about the proteins that repress β-globin transcription. Identification of proteins involved in repression may have clinical importance since repression of the β^S-globin gene would be beneficial in SCA and, if there is a reciprocal elevation of γ-globin expression, repressors may be useful in other hemoglobinopathies as well.

Previously, we identified two silencer regions upstream of the β-globin gene, silencers I and II and two proteins binding to those silencers, termed BP1 and BP2 [1]. BP1 binds to silencer I DNA at −530 bp and to

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silencer II DNA at −300 bp, while BP2 binds to silencer II at −270 bp. Relief from repression is seen when either the −300 BP1 binding site or the −270 BP2 binding site is inactivated by site directed mutagenesis, demonstrating the functional importance of these two binding sites in repression [2].

In this study, we chose to focus on silencer I because binding of BP1 protein at −530 bp may be specifically relevant to SCA. In SCA there are five haplotypes associated with the mutant β^S-globin gene [3–5]. Patients with the Indian haplotype exhibit mild symptoms of the disease, in contrast to patients with the Bantu haplotype, who are severely affected [6–8]. There is also a polymorphism in the BP1 binding site at −530 bp which varies among the five haplotypes [9]. We found an inverse correlation between the binding affinity of BP1 at this −530 bp site and the severity of SCA, i.e., BP1 binds five to six times more tightly to the DNA having the Indian haplotype sequence at −530 bp than to DNA with the reference sequence at that site, and two to three times more weakly to DNA with the Bantu sequence at −530 bp than the reference sequence [10–12]. These observations suggest that BP1 and possibly other proteins binding to this region may be important in determining the severity of SCA.

For this reason, and because of the potential therapeutic benefits of repression of the β-globin gene, mentioned above, we used an oligonucleotide probe to isolate proteins binding to the −530 region of silencer I. Using this approach, we cloned two types of cDNAs, which encode either HMG-I or HMG-Y, two low molecular weight, nonhistone proteins produced by alternative splicing [13]. HMG-I(Y) serve as architectural proteins in the formation of transcription complexes that regulate several genes, including IFN-β, IFN-γ, and HLA-DRA [14–18]. HMG-I(Y) can bind to the minor groove of DNA, causing DNA bending or, in the case of IFN-β, reversing the intrinsic bend of the DNA [19], thereby allowing transcription factors to bind in the major groove. We show that binding of HMG-I(Y) causes DNA flexure in the −530 β-globin region, suggesting it may also play an architectural role in repression through silencer I and facilitate binding of repressor proteins. To better understand the relationship between HMG-I(Y) and β-globin expression, the pattern of RNA expression of HMG-I(Y) was examined in over 50 human tissues and cell lines. Based on our findings and those of others, we propose a model for repression of the β-globin gene which integrates data on HMG-I(Y) binding, DNA bending and looping upstream of the β-globin gene, and the positive and negative regulatory regions found upstream of this important gene.

MATERIALS AND METHODS

Cloning of HMG-I(Y) cDNA

A K562 λgt11 cDNA expression library (Clontech, Inc., Palo Alto, CA) was screened with an oligonucleotide probe containing the BP1 binding site in silencer I DNA, including sequences from −555 to −502 bp upstream of the β-globin gene. Screening was as described [20], with several modifications. Filters were prehybridized in binding buffer [10] containing 5% Carnation instant dry milk at 4°C for 30 min, then hybridized in the same buffer for 2 to 12 hr at 4°C with shaking. Positive plaques were purified and subjected to three additional rounds of screening.

Electrophoretic Mobility Shift Assay (EMSA)

The binding reaction of 25 μl included 500 pg of purified protein or 250 ng of nuclear extract added to binding buffer (10 mM Tris, pH 7.5, 50 mM NaCI, 5% glycerol, 1 mM DTT, 1 μl of 1 mg/ml BSA, 0.15 μg poly[dG-dC], and 5,000–10,000 cpm of labeled probe). Incubation was for 20 min at room temperature. Nuclear extracts were prepared according to Dignam et al. [21]. Protein extracts from lysogens were prepared as described [22]. Competitors were added with the probe. They included the following oligonucleotides:

−530 Reference 5′ TGTATATATACACATATATATA-
TATATTTTTTTCTTTTCTTACCAGAAGGTTT 3′
−530 Indian 5′ TGTACATATACACATATATA-
TATATATATATTTTTTCTTTTCTTACCAGAAG-
GTTT 3′
−300 beta 5′ TTCTTATTTGTGTAATAAGAAAAT-
TGGGAAAACGATCTTCAATATGCTTACCAA-
GCTG 3′
−530 delta 5′ TTCTTTTAATGGATATTTATTTCA-
ATATAATAAAAAATTAGAGTTTTA 3′

Glutathione S-Transferase (GST)-HMG-I Fusion Protein

Full-length HMG-I cDNA was cloned in-frame into the *Eco* R1 site located downstream of the GST gene in the pGEX-4T-1 vector (Pharmacia, Piscataway, NJ). The fusion protein was purified using a GST Purification Module (Pharmacia).

Circular Permutation Assays

Annealed and phosphorylated oligonucleotides containing the reference and Indian haplotype of silencer I were blunt-end ligated into the unique *Xba* I site of pBEND2 [23]. This vector was the kind gift of Dr. Sankar Adhya. Seven different digests (*Mlu* I, *Bgl* II, *Xho* I, *Eco* RV, *Sma* I, *Ssp* I, *Bam* HI) were then performed for both the reference and Indian haplotype DNAs. The resulting 14 bands were of equal length but contained the

cloned oligonucleotide at a different position relative to the ends of the restriction fragment.

Northern Blot Analysis

RNA was isolated using RNeasy and Oligotex kits (Qiagen, Valencia, CA). Ten micrograms of total RNA or 2 μg of mRNA was electrophoresed per lane, then transferred to Hybond N (Amersham, Arlington Heights, IL) by capillary action. RNA was crosslinked to the membrane using a Stratolinker (Stratagene, La Jolla, CA), and the filter was hybridized using a sandwich method [24]. The probe was a 950 bp fragment containing HMG-I cDNA obtained by digestion of λgt11 with *Eco* RI. Analysis of a dot blot containing 50 tissues (Clontech) was also performed using the sandwich method. The probe was labeled by random priming and 2×10^6 cpm/ml were used in the hybridization reaction, which was performed overnight at 65°C. After washing, the blot was exposed to X-ray film for 2.5 days. An adult and a fetal multiple tissue blot were purchased from Clontech. For RNA from primary erythroid cultures, comparable amounts of ribosomal RNA were used in each analysis, representing a comparable number of cells [25].

Erythroid Cell Cultures

Erythroid progenitors were obtained by culturing peripheral blood (PB) using a previously described two-phase liquid culture procedure [25,26]. Mononuclear cells were isolated from the buffy coat of PB obtained from normal volunteers by centrifugation over Lymphocyte Separation Medium (Organon Teknika, Durham, NC). These cells were incubated at 37°C in 5% CO_2 for 5–7 days in phase I medium [α-minimal essential medium (α-MEM)] (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (HyClone, Logan, UT), 1.5 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml cyclosporin A (Sandoz Pharmaceuticals Corp., East Hanover, NJ), and 10% conditioned medium from the 5637 bladder-carcinoma cell line [27]. Subsequently, nonadherent cells were washed, then reincubated for the indicated number of days in phase II medium (α-MEM supplemented with 30% fetal calf serum (FCS), 1.5 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% deionized bovine serum albumin (Sigma, St. Louis, MO), 1×10^{-5} M 2-mercaptoethanol, 1×10^{-6} M dexamethasone (Sigma), 0.3 mg/ml holo-transferrin (Sigma), 10 ng/ml human recombinant stem cell factor (Sigma), and 1 U/ml human recombinant erythropoietin).

RESULTS

HMG-I(Y) Binds to Silencer I DNA

A multimerized oligonucleotide containing the −530 bp binding site recognized by BP1 activity was used as the probe to screen a λgt11 cDNA expression library made from K562 cells. K562 cells are human erythroleukemia cells that, when induced by hemin, express embryonic and fetal globin genes, but do not express the adult β-globin gene, although the gene is structurally normal and can be transactivated [28,29]. K562 cells were chosen as the cDNA source because silencer I is able to negatively regulate a reporter gene in K562 cells, indicating the presence of repressor proteins able to bind to silencer I DNA [1]. One million plaques were screened, resulting in the isolation of three cDNAs. Sequencing identified the cDNAs as HMG-I or HMG-Y, known isoforms of HMG-I(Y) (data not shown).

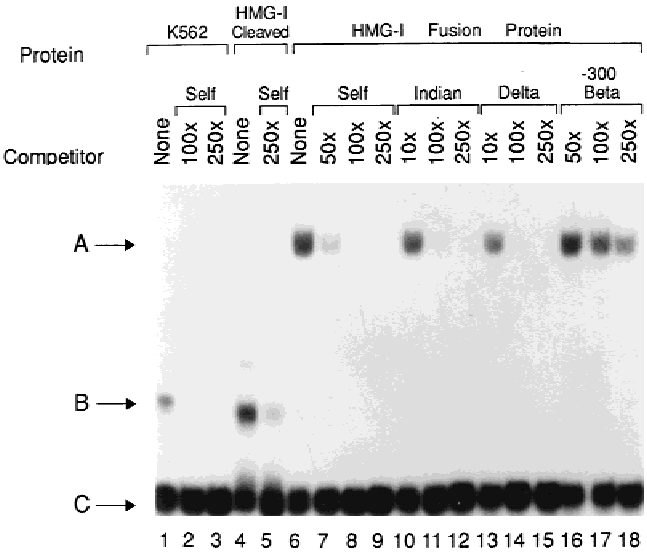

Fig. 1. Binding specificity of HMG-I. EMSA analysis was used to determine the sites to which HMG-I binds. The protein source was a nuclear extract from K562 cells in lanes 1–3, HMG-I/GST fusion protein after cleavage with thrombin in lanes 4 and 5, and uncleaved HMG-I/GST fusion protein in lanes 6–18. The probe was the oligonucleotide at −530 bp upstream of the β-globin gene which was used to clone HMG-I. Competitors were self (unlabeled −530 bp), Indian (−530 β/Indian), delta (−530 δ), and −300 beta (−300 β). The band at position A is due to binding of GST/HMG-I to the probe; the band at B is due to binding of native HMG-I protein or thrombin cleaved HMG-I protein; and the band at C denotes the position of the unshifted probe.

Since HMG-I and HMG-Y expressed in plaques exhibited identical binding patterns using a variety of labeled DNA probes (data not shown), additional binding experiments were performed using electrophoretic mobility shift competition assays with HMG-I (Fig. 1). The probe was the same oligonucleotide used in cloning HMG-I(Y) called −530 β. It was first incubated with nuclear extract from K562 cells, giving a single shifted band, denoted by B (lanes 1–4). This band represents binding of the native protein to the probe. Since HMG-I(Y) has a molecular weight of only about 10,000 [30],

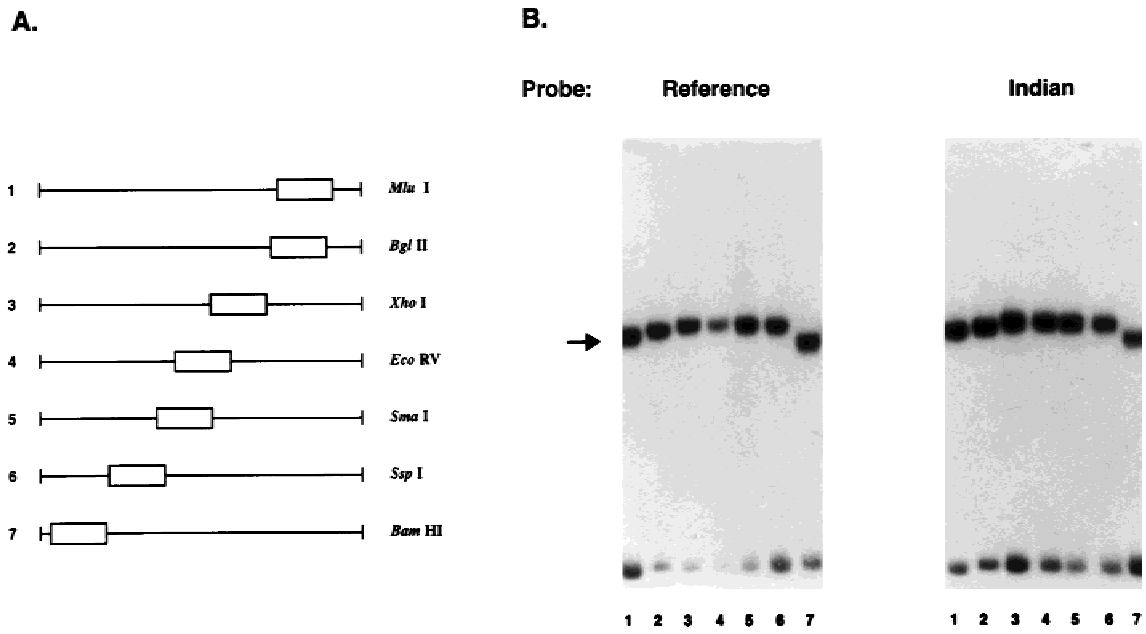

Fig. 2. Binding of HMG-I causes DNA flexure. (A) DNA restriction fragments used in the circular permutation assays. The open box indicates the position of the oligonucleotide containing the HMG-I(Y) binding site relative to the ends of each restriction fragment. (B) Circular permutation assays. The assay on the left was performed using restriction fragments containing the reference sequence at −530 bp, while the assay on the right was performed using restriction fragments containing the Indian/Arabo haplotype sequence at −530 bp. The arrow indicates the position of the shifted band. The numbers below each lane designate the restriction fragment used as the probe, corresponding to the numbers in panel A.

the shifted band is near the unshifted probe, denoted by C. Band B is specific, since it is competed by an unlabeled probe used as a cold DNA competitor (self, lanes 2 and 3). The reason that there is no shifted band corresponding to BP1 binding in this assay is that the use of poly(dG-dC) as a nonspecific competitor strongly favors HMG-I(Y) binding (M.C. and P.B., unpublished observations). Conversely, in the assays used earlier to identify BP1, we used poly (dl-dC), which effectively blocks HMG-I(Y) binding. A purified fusion protein of HMG-I and GST was produced, and free HMG-I protein was generated from this GST-HMG-I fusion protein by cleavage with thrombin, which leaves only two amino acids from GST fused to HMG-I. The cleaved protein was incubated with the probe, resulting in a shifted band near position B (lane 4) which was competed by unlabeled probe (lane 5). Binding assays in lanes 6 to 18 were performed using the uncleaved GST-HMG-I fusion protein that, because of its greater molecular weight, retards the probe to position A. Three of the cold competitors competed with HMG-I for binding to the −530 β probe: Self (unlabeled probe) DNA (lanes 7–9), an oligonucleotide containing the Indian haplotype sequence at −530 bp (lanes 10–12), and oligonucleotide containing the BP1 binding site at −530 bp upstream of the δ-globin gene [31] which is within a negative regulatory region [32] (lanes 13–15). Although the DNA containing the binding site for BP1 at −300 bp appeared to compete weakly with HMG-I for binding (lanes 16–18), in other experiments we did not observe competition with this DNA. No apparent difference was seen in the affinity of HMG-I(Y) for the reference sequence (Self DNA) compared with the Indian haplotype sequence (lanes 7–12). In a number of other similar experiments, no binding was observed to negative control sequences farther upstream of the β-globin gene, at −580 bp (data not shown).

HMG-I Binding Causes DNA Flexure at −530 bp

Since HMG-I(Y) is capable of bending DNA [14–18], we tested the ability of our cloned HMG-I(Y) to cause bending or flexure of the DNA in silencer I. A circular permutation assay, developed to detect distortion of DNA by binding of proteins [33,34], was used to examine this possibility (Fig. 2). A number of proteins have been shown to exhibit the ability to bend or distort DNA using this assay (see, for example, references 35–37). An oligonucleotide bearing the wild type (reference) or Indian haplotype sequence of silencer I was cloned into the unique *Xba* I site of the vector pBend 2, a vector designed for use in the circular permutation assay [23]. Flanking the unique cloning site on both sides are duplicated restriction sites (*Mlu* I, *Bgl* II, *Xho* I, *Eco* RV, *Sma* I, *Ssp* I, and *Bam* HI). Results of cleavage with each enzyme are shown diagramatically, with the open box

representing the position of the cloned oligonucleotide containing the HMG-I(Y) binding site (Fig. 2A). The restriction fragments were each end-labeled for their use as probes in an EMSA, seven fragments containing the reference sequence and seven fragments containing the Indian haplotype sequence. Each fragment was incubated with GST-HMG-I fusion protein and analyzed by EMSA (Fig. 2B). The arrow denotes the shifted band, and the band at the bottom of the gel is free probe. The number of each lane indicates the restriction fragment used as the probe.

If HMG-I bends or distorts the DNA, the migration of a fragment in the gel will vary depending on where in the DNA the flexure occurs [23,33,34,38]. When flexure occurs in the middle of the fragment, the DNA migrates the slowest, while flexure at the ends increases its mobility. If there is no flexure, all fragments migrate with the same mobility. The results demonstrate bending/distortion of both the Reference sequence and the Indian haplotype sequence by HMG-I. There is no indication of intrinsic flexure of either oligonucleotide since the position of the unbound probe does not change when the position of the HMG-I(Y) binding site is moved within the oligonucleotide (lanes 1–7 and lanes 8–16). The circular permutation assay was also performed using crude protein extract from K562 cells, with similar results (data not shown).

HMG-I(Y) Expression Is Ubiquitous

Since HMG-I(Y) may be involved in repression of the β-globin gene through silencer I, HMG-I(Y) expression might be ubiquitous in tissues not expressing β-globin. Surprisingly, expression of HMG-I(Y) has not been well characterized in human tissues. Therefore, we performed extensive Northern blot analysis to determine the tissue distribution of HMG-I(Y) mRNA. Preliminary experiments using five adult tissues (lung, liver, muscle, kidney, and pancreas), four fetal tissues (brain, lung, liver, and kidney), and placenta showed a single band of 2000 bp, the mRNA size expected for HMG-I(Y) [13] (data not shown). The only tissue that showed no HMG-I(Y) expression was adult liver. Next, we probed a commercially obtained Northern RNA dot blot (Clontech) containing 50 tissues (Fig. 3 and Table I). Since there was no detectable background using the sandwich method of Northern blotting (described in Materials and Methods), even after two weeks of exposure of the film, we are confident that the hybridization signals observed after three days of exposure were due to the presence of HMG-I(Y) RNA. Using this assay, HMG-I(Y) expression was detected in all human tissues except the ovary and adult liver. Expression varied from strong to very weak. Tissues most highly expressing HMG-I(Y) include testis, small intestine, thymus, fetal liver, and fetal thymus. There results are in agreement with the Northern blots described above, which also showed very high expression in fetal liver and undetectable expression in adult liver. In the controls, described in the legend to Figure 3, the HMG-I(Y) probe hybridized only to the human nonrepetitive DNA (row H, lanes 7 and 8), verifying the specificity of hybridization.

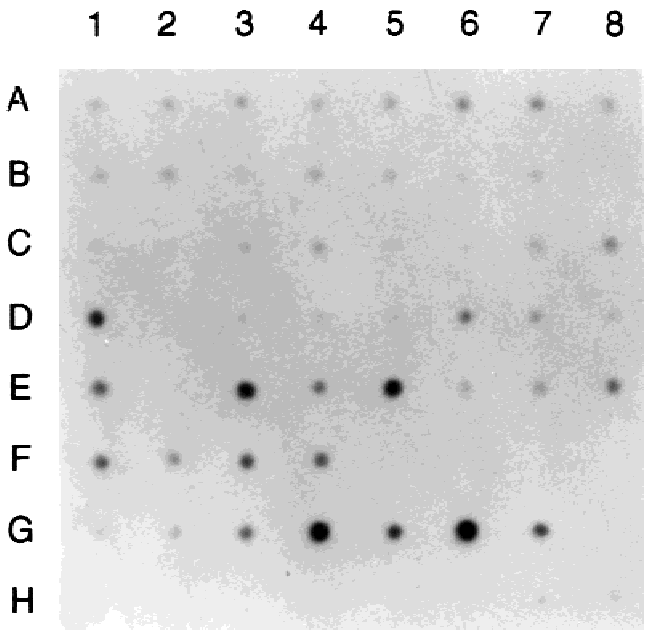

Fig. 3. Northern blot of HMG-I(Y) in 50 tissues. Northern blot analysis was performed using a partial HMG-I cDNA as a probe. The letters and numbers indicate the position of different samples. Refer to Table I for a description of the location of RNA from different tissues, located in rows A–G. Row H contains the following controls: yeast RNA (lane 1); yeast tRNA (lane 2); *E. coli* RNA (lane 3); *E coli* DNA (lane 4); poly r(A) (lane 5); human C_0t 1 DNA (lane 6); human DNA, 100 ng (lane 7); and human DNA, 500 ng (lane 8). Positions B8, F5–8, and G8 do not contain samples.

To define HMG-I(Y) expression in hematopoietic cells, mRNA from a variety of hematopoietic cell lines was analyzed (Fig. 4). HMG-I(Y) is expressed in all of the cell lines examined, including erythroid K562 and HEL cells (lanes 1 and 2), monocytic THP-1 and U937 cells (lanes 3 and 5), monocytic/granulocytic HL60 cells (lane 4), and megakaryocytic MEG-01 cells (lane 6). The β-actin loading control is shown below. Note that two bands are observed in each lane. These bands are of 2000 bp and 3800 bp, and have been reported previously only in K562 cells [39].

HMG-I(Y) Is Reduced During Erythroid Differentiation

If HMG-I(Y) is required for repression of adult globin genes, as we hypothesize, its mRNA level may diminish during erythroid differentiation. Precedent for down-regulation of HMG-I(Y) exists in chickens, where there is reduced HMG-I(Y) in erythroid cells of 14-day chick embryos compared with 5-day chick embryos, whereas

TABLE I. Expression of HMG-I(Y) in Multiple Tissues*

+++[a,b]	++	+	+/−	−
Testis (D1)	Frontal lobe (A6)	Whole brain (A1)	Substantia nigra (B3)	Ovary (D2)
Small intestine (E3)	Hippocampus (A7)	Amygdala (A2)	Heart (C1)	Liver (E2)
Thymus (E5)	Colon (C4)	Caudate nucleus (A3)	Aorta (C2)	
Fetal liver (G4)	Stomach (C8)	Cerebellum (A4)	Bladder (C5)	
Fetal thymus (G6)	Thyroid (D6)	Cerebral cortex (A5)	Uterus (C6)	
	Salivary gland (D7)	Medulla oblongata (A8)	Adrenal gland (D5)	
	Kidney (E1)	Occipital lobe (B1)		
	Spleen (E4)	Putamen (B2)		
	Lymph node (E7)	Temporal lobe (B4)		
	Bone marrow (E8)	Thalamus (B5)		
	Appendix (F1)	Subthalamic nucleus (B6)		
	Trachea (F3)	Spinal cord (B7)		
	Placenta (F4)	Skeletal muscle (C3)		
	Fetal kidney (G3)	Prostate (C7)		
	Fetal spleen (G5)	Pancreas (D3)		
	Fetal lung (G7)	Pituitary gland (D4)		
		Mammary gland (D8)		
		Peripheral leukocyte (E6)		
		Lung (F2)		
		Fetal brain (G1)		
		Fetal heart (G2)		

*The loading position of each RNA on Figure 3 is denoted by the letter and number in parenthesis.
[a]The relative levels of expression are shown, with +++ indicating the highest expression.
[b]Row H contains the following controls: yeast total RNA (H1); yeast tRNA (H2); *E coli* rRNA (H3); *E. coli* DNA (H4); poly r(A) (H5); human C_0t 1 DNA (H6); human DNA, 100 ng (H7); and human DNA, 500 ng (H8).

there is no detectable HMG-I(Y) in circulating red cells from adult animals [40]. To test this possibility in human cells, HMG-I(Y) mRNA expression was analyzed during differentiation of primary adult erythroid cells grown in a two phase liquid culture system (see Materials and Methods). After stimulation with erythropoietin in phase II, β-globin expression increases until it peaks on day 13 [41]. Therefore, we chose to look at HMG-I(Y) expression on day 13 and, for comparison, on day 6, when β-globin expression is still rising. In Figure 5, HMG-I expression is very high in K562 cells (lane 1) relative to day 6 (lane 2). On day 13, no HMG-I(Y) was detected in either of two samples (lanes 3 and 4). The samples in lanes 2 and 3 were from the same culture, giving a direct comparison of expression of days 6 and 13. Therefore, HMG-I(Y) expression decreases in primary peripheral blood cells during the course of erythroid differentiation.

DISCUSSION

In this study, we demonstrate that HMG-I(Y) binds specifically to negative regulatory regions upstream of both the adult δ- and β-globin genes. In other systems, HMG-I(Y) serves as an accessory factor that alters DNA structure in such a way as to allow formation of regulatory protein complexes [14–19]. For example, HMG-I(Y) is essential for induction of the IFN-β, IFN-γ, and HLA-DRA genes [14–18]. Although HMG-I(Y) is required for repression of the immunoglobulin heavy chain germ-line ε promoter, no structural role has yet been demonstrated by its binding [42].

We determined that binding of HMG-I(Y) induces structural alterations in the DNA within silencer I of the β-globin gene. Circular permutation assays were used to demonstrate that HMG-I(Y) causes flexure or bending in an oligonucleotide containing the HMG-I(Y) binding site from silencer I, whether this sequence was from the reference or Indian haplotype. Although we did not detect intrinsic bending of the oligonucleotide containing the HMG-I(Y) site, there may be intrinsic bending in a larger region containing the sequences of the oligonucleotide. Using a 263 bp sequence (−664 to −401 bp) in the circular permutation assay, bending was detected centered approximately 60 bp upstream of the BP1 binding site, which was not included on our oligonucleotide [43]. A 750 bp fragment containing the reference or Indian haplotype DNA sequence at −530 bp was also tested for curvature, with the finding that both sequences exhibit intrinsic curvature [44]. Taken together, these data suggest that the binding of HMG-I(Y) may introduce a second bend downstream from the intrinsic bend, enhance the intrinsic bend, or straighten the bend.

Our findings show that two proteins, BP1 and HMG-I(Y), bind to a single oligonucleotide containing sequences centered at −530 bp upstream of the adult δ-globin gene and to an oligonucleotide containing sequences centered at −530 bp upstream of the adult β-globin gene [1,27], indicating that binding of both proteins may be

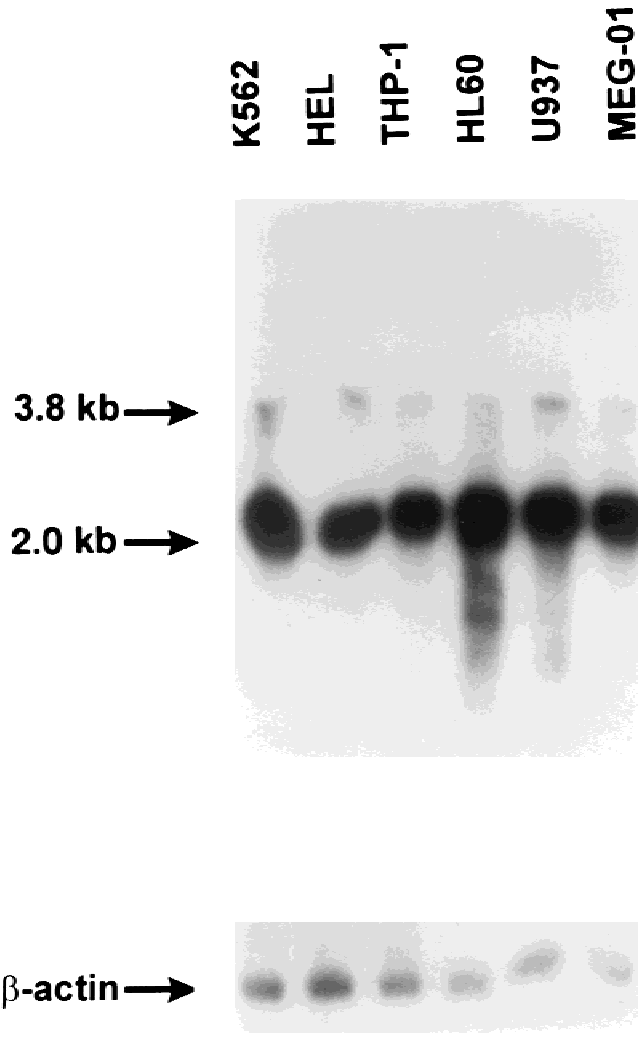

Fig. 4. Expression of HMG-I(Y) in hematopoietic cell lines. Northern blot analysis was performed using 2 μg of purified mRNA in each lane from the cell line indicated. The probe was the same as in Figure 4. The arrows indicate the sizes of the two bands observed. The loading control, β-actin, is shown at the bottom.

important in the known coregulation of the β- and δ-globin genes. The fact that HMG-I(Y) does not bind to the BP1 binding site in silencer II indicates that HMG-I(Y) binding is not obligatory for BP1 binding, however.

Binding of HMG-I(Y) and BP1 at the −530 polymorphic site in silencer I is of clinical interest since SCA patients with the Indian haplotype sequence exhibit significantly fewer complications than patients with the reference sequence, as discussed in the Introduction. In earlier experiments, we presented evidence that BP1 protein binds with higher affinity to DNA having the Indian haplotype sequence compared with the reference sequence [11]. HMG-I(Y) could theoretically be involved in this difference in binding affinity by altering the degree of DNA flexure at the Indian haplotype site relative to the reference sequence, affecting the binding of BP1.

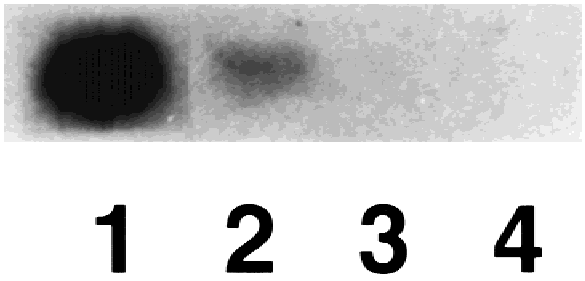

Fig. 5. Expression of HMG-I(Y) during erythroid differentiation. Total RNA was extracted from erythroid progenitors undergoing terminal differentiation. Lane 1, K562 cell control; lane 2, day 6, culture A; lane 3, day 13, culture A; lane 4, day 13, culture B.

For this reason, we tested DNA flexure of these two sequences by HMG-I(Y) using circular permutation assays. No difference was detected in the degree of flexure between the reference and Indian haplotype sequences, either by inspection or by plotting the changes in relative mobility shown in Fig. 2B (data not shown). Using the standards of Thompson and Landy [38], we estimate the angle of flexure for both sequences to be 36°. Therefore, this idea does not appear likely. Alternatively, HMG-I(Y) itself may bind with different affinities to the Indian and reference DNAs, thereby affecting BP1 binding affinity. However, EMSA analysis demonstrated that HMG-I(Y) exhibits a similar binding affinity for the Indian and reference sequences (Fig. 1). In addition, BP1 protein binds to both the reference and Indian haplotype sequence with different affinities in the absence of HMG-I(Y) [45]. These data suggest that the enhanced binding of BP1 to the Indian sequence is an intrinsic property of BP1, although both the binding affinity and the changes in DNA structure induced by HMG-I(Y) may be influenced by sequences outside of the oligonucleotide used in these assays.

The expression of HMG-I(Y) is ubiquitous, being expressed in 48 tissues and absent only in adult liver and ovaries. This may indicate that it is universally required for repression of the β-globin gene, that it is present in those cells to regulate other genes, or both. The abundant expression of HMG-I(Y) in fetal liver suggests it could be a regulator of β-globin in hematopoietic tissues since fetal liver is the site of synthesis of fetal hemoglobin. This idea is consistent with the observation that HMG-I(Y) mRNA was present in six human hematopoietic cell lines, none of which expresses β-globin. If HMG-I(Y) is required for repression of β-globin expression, its absence is not sufficient to allow induction of β- or δ-globin expression, as demonstrated in adult liver cells and ovaries.

Two species of HMG-I(Y) mRNA, 3800 bp and 2000 bp, were present in all six hematopoietic cell lines. The 3800 bp mRNA was previously observed in K562 cells

[39]. Although the functional significance of the 3800 bp band is unknown, the 3′ end of the 3800 bp and 2000 bp messages differ. By contrast, only the 2000 bp mRNA was observed in HuT78 (mature T lymphocyte) cells and in human B lymphocytes [13,39,46]. The 3800 bp mRNA thus may have a function related to myeloid hematopoiesis.

A plausible model of negative regulation of the β-globin gene must take into account the following observations: 1. there are intrinsic bends in the DNA of both silencer I and II [43,47]; 2. HMG-I(Y) binding introduces additional DNA flexure in silencer I; and 3. HMG-I(Y) also binds to a GATA-1 site at −205 bp upstream of the β-globin gene [48], within a region we have determined to be a positive control region (P.B., unpublished data). The function of HMG-I(Y) binding at that site is unknown. We propose that HMG-I(Y) binding facilitates binding of repressors to silencer I and that there is interaction between proteins binding to silencers I and II, preventing transcription, perhaps by DNA looping. There is evidence that looping occurs upstream of the β-globin gene [49]. In this model, decreased HMG-I(Y) binding would occur upon erythroid differentiation, relieving repression at the −530 site of the β-globin gene and allowing transcriptional activation involving the GATA-1 binding site at −205 bp. In support of this hypothesis, measurement of HMG-I(Y) mRNA in primary cultured erythroid progenitors showed gradual down-regulation of HMG-I(Y) during erythroid differentiation.

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